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Germinal Cell Mutagenesis in Specially Designed Maize Genotypes

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We have used three inbreds of *Zea mays* in our *in situ* and laboratory studies in environmental mutagenesis. Inbred W22 plants homozygous for *wx-C* were used in a study to detect the possible mutagenic properties of 32 pesticides or combination of pesticides under modern agricultural conditions. The large numbers of pollen grains analyzed and the ease in detecting mutant pollen grains enabled us to treat the experimental plants with field recommended rates of pesticides. In a current study we are evaluating the possible mutagenicity of Chicago municipal sewage sludge. We are measuring the frequency of mutant pollen grains in inbred M14 at both the *wx-C* and *wx-90* heteroalleles. These plants were exposed to various concentrations of municipal sewage sludge under field conditions. We have inbred Early-Early Synthetic for five generations and tested this inbred with known mutagens. Early-Early Synthetic is a rapidly maturing inbred growing from kernel to anthesis in approximately 4 weeks and attaining a height of approximately 50 cm. Plants of this inbred have been chronically treated with ethylmethanesulfonate (EMS) or maleic hydrazide (MH) under laboratory conditions and forward mutation at the *wx* locus was measured in the pollen grains. EMS and MH were mutagenic at concentrations of 1 μM and 10 nM, respectively. The concentrations of EMS and MH were calibrated in Early-Early Synthetic to a linear increase in the frequency of forward mutant pollen grains. The construction of a maize monitor for environmental mutagens is currently in progress. This assay will measure forward or reverse mutation at the *wx* locus in pollen grains, point mutation in somatic cells and will incorporate a cytogenetic endpoint in root-tip cells.

Introduction

Historically, plants have been used as indicator organisms in studies on mutagenesis in higher eukaryotes. Plant systems have a variety of well defined genetic endpoints including alterations in ploidy, chromosome and chromatid aberrations, micronuclei, sister chromatid exchanges, and specific locus and multilocus assays in both nuclear and cytoplasmic genomes. Plant genetic assays have inherent advantages as indicators of environmental mutagens and are the only systems currently in use as *in situ* monitors of polluted air (*1-3*), polluted water (*4*), and agricultural pesticides (*5, 6*). An excellent description of the advantages of plant systems as monitors of environmental mutagens has been written by Nilan (*7*).

An environmental mutagen is a physical or chemical agent released into the environment that can alter the genome or the proper functioning of the genome. The presence of such genotoxic agents in the environment is a serious threat to the public health (*8-13*). Depending upon the ontogenetic stage of an organism an environmental mutagen may exert teratogenic effects, affect the aging process, induce mutations that involve germinal cells (*14-16*), precipitate coronary disease (*17*) or cause mutations that lead to the neoplastic transformation of somatic cells (*12, 18-22*). Genetic assays have been developed to determine the mutagenic properties of chemicals (*23*). The approach to reduce the impact of environmental mutagens upon the public health is simply to reduce the exposure of people to such agents. The role of regulating the release of toxic chemicals into the environment is a responsibility of the U.S. Environmental Protection Agency. The enforcement powers of such regulation are derived from federal statutes such as the Toxic Substances Control Act, the Resource Conservation and Recovery Act and the Clean Air and Clean Water Acts. Environmental mutagens are defined as toxins (genotoxins) and thus are under the control of several federal and state agencies. However, a

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battery of good and quantitative genetic assays must be used to adequately determine the genotoxic properties of chemicals, combinations of chemicals and complex environmental pollutants. The work that we present in this paper is an attempt to devise and calibrate an assay in a higher eukaryote that would be useful in a battery of genetic assays to detect the presence of mutagens in the environment.

A series of problems arises when working with higher eukaryote species. The resolution of a genetic endpoint is usually lowered when one proceeds from prokaryotes (bacteria) to lower eukaryotes (fungi) to higher eukaryotes (rodents, humans, angiosperms). As the complexity of the genetic information and the architecture of the chromosome increases, the size of the population routinely analyzed for each assay decreases. Thus the concentration of the test chemical or complex mixture must be increased and problems due to the degree of tolerance for the test agent may arise. Another difficulty is the treatment protocol. Most genetic assays involve acute exposure; however, people are usually exposed to environmental mutagens under chronic conditions.

The objectives of this study are: to define and test a point mutation assay that would have a high degree of genetic resolution based on a higher eukaryote (*Zea mays*), to investigate the use of this assay under both laboratory and *in situ* conditions, to calibrate a genetic endpoint with known mutagens under acute and chronic exposure regimens, and to construct special lines of maize that carry a number of different genetic endpoints for use as monitors for environmental mutagens.

The *waxy* Locus of *Z. mays*

We chose mutation at the *waxy (wx)* locus in maize as the genetic endpoint because it is well defined and it is easy to detect mutations in both the kernel and pollen grain. The use of pollen grains as genetic indicator organisms provides a high degree of genetic resolution. Also a tassel growing within a sporophyte may be considered analogous to a suspension culture growing with time. Therefore this system is suitable for tests involving chronic treatment of sporophytes with mutagens. Finally the tassels can be stored and the *wx* locus assayed for mutation in the pollen grains at a time removed from the end of the treatment protocol or anthesis. This advantage allows for the collection of large numbers of samples during a growing season and the analysis of pollen grains at any convenient time.

Near the beginning of the twentieth century an altered kernel characteristic in maize was introduced into the United States from China that was different from the American varieties of floury, sweet, flint, or pop. This novel variety was termed "waxy" because the endosperm had the appearance of hard wax (*24*). Genetic studies confirmed that the *waxy* allele was recessive to starchy (*Wx*) and *wx* segregated in the F_2 generation as a Mendelian monohybrid (*25*). The waxy characteristic was due to a mutation that altered the composition of the starch in the endosperm of the kernel. In waxy kernels, the starch of the endosperm contains only amylopectin, while in kernels carrying the dominant allele, *Wx*, the endosperms contain starch composed of a mixture of amylopectin and amylose (*26*, *27*). Because of the presence of amylose the endosperms of kernels carrying the *Wx* allele stain a blue-black color when reacted with iodine. When an iodine solution is reacted with endosperms of kernels homozygous for *wx* a red color is produced.

The enzymatic differences inherent in the endosperms of homozygous *wx* maize kernels as compared to those kernels carrying the dominant *Wx* allele were investigated by Nelson and Rines (*28*). Homozygous *wx* kernels contain the same amount of starch as starchy kernels. However, the waxy kernels do not have the uridine diphosphate glucose transferase system. Since the starch in waxy kernels is entirely composed of amylopectin (*27*), this carbohydrate must be synthesized by a different biochemical pathway than the branching of amylose formed via uridine diphosphate glucose transferase (*28*).

The use of the iodine test provided an early, rapid and accurate chemical assay for a genetic characteristic. It was soon discovered that the *wx* phenotype could be detected by the iodine test in the microgametophytes (pollen grains). Pollen grains are functional haploids and in a heterozygous plant both alleles segregate according to Mendel's first law (*29*, *30*). Furthermore, the data indicate that a single gene and its alleles can be similarly expressed in both the sporophytic and gametophytic generations.

Since the *wx* allele can be detected in single pollen grains it was suggested that this system be used in the study of the genetic fine structure of a locus in a higher eukaryote (*31*). These suggestions were based on the then recent discoveries that redefined the classical structure of the gene (*32*). The increase in genetic resolution, however, required very large populations (*33*) and a population in excess of 10^5 is usually impossible to analyze in higher eukaryotes. Maize pollen grains were suited to the problem of population size because great numbers could be analyzed rapidly. Also, at that

time a few independently occurring *wx* mutations (heteroalleles) were collected and were available for *cis-trans* tests (*34*). A map of the *wx* locus illustrating the position of four mutational sites, *wx-H21*, *wx-C*, *wx-90*, and *wx-B* from Nelson, (*35*) is presented in Figure 1.

The starch type of a pollen grain is controlled by the genetic constitution of that pollen grain, not by the parental sporophyte. Thus, a genetic reversion of *wx* to *Wx* can be detected by scoring for pollen grains from plants that are homozygous *wx* and that stain a dark blue-black color when subjected to an iodine test (*34*, *36*). Intragenic recombination between two different mutations at the *wx* locus can be analyzed by scoring for *Wx* pollen grains from plants that are intercrosses of lines representing the different heteroalleles (*34*,*36*).

To date, 31 different heteroalleles have been mapped at the *wx* locus (*35*). Five of these mutations are controlling element alleles, *wx-m1*, *wx-m-6*, *wx-m-8*, *wx-B3*, and *wx-B4*. These heteroalleles are of interest because they possess functional *Wx* alleles that are prevented from functioning by the controlling element (*37*). Finally, the phenomenon of gene conversion (nonreciprocal intragenic recombination) has been reported at the *wx* locus (*38*).

The studies cited in this paper deal with the use of the *wx* locus as an indicator of mutation and the analysis of dose-response relationships. Other studies in which the primary objective was the induction of new *wx* alleles and their genetic analysis are not discussed.

In maize both acute and chronic treatments by chemical and physical mutagens have been used. This classification is justifiable in the biological sense as well as in terms of the duration of mutagen treatment because of the different cell populations at risk, the number of cell generations and the morphogenetic processes involved. Experiments conducted by Eriksson and others (*39-44*) on acute and chronic exposure of maize to radiation will be used as examples of the differences inherent in the treatment regimes. Forward mutation at the *wx* locus in pollen grains was the genetic endpoint.

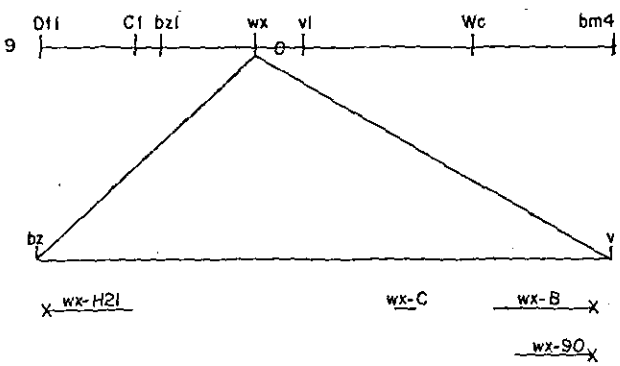

FIGURE 1. Location of the *wx* locus and four heteroalleles on chromosome 9 in *Zea mays* [From Nelson (*35*)].

In experiments that involve acute exposures to a mutagen two approaches are usually used, constant time duration at varying doses, and constant dose for various durations. Eriksson (*39*) treated maize plants that were at the microsporocyte stage of development. Most of the developing meiocytes of the plants were at prophase I. The plants were exposed to rates of γ-radiation of 1.5 to 200 R/hr. The radiation exposures ranged from 0 (control) to 400 R during a 2 hr period. All the tassels were collected and fixed on the same day following treatment and it was assumed that all of the anthers were in the same stage of development when treated. The control frequency of forward mutant pollen grains was 3.7×10^{-4} while an exposure of 3 R increased the frequency of mutants to 5.7×10^{-4} and 400 R increased it to 6.0×10^{-3}. The frequency of mutant pollen grains increased in a linear manner for exposures through 200 R.

Under a chronic treatment regimen the sporophyte is subjected to mutagenic treatment during a major portion of its life cycle. Each cell generation is exposed to a limited quantity of the mutagen and it is assumed that a constant rate of mutagen enters the target cells (i.e., the anther). A number of other factors exists that can influence the genetic response when the plant is chronically treated with a mutagen. A mutational event may occur at any ontogenetic stage and yield mutant pollen grain clusters that vary in size inversely related to the development of the plant. The number of germline cells varies at different stages during ontogeny. The probability of inducing a scorable mutation at the *wx* locus in a germline cell increases as a plant develops; however, the number of mutant pollen grains that result from such a mutational event decreases. Finally, the frequency of the induction of mutation varies as a function of the dose of a mutagen. Under chronic conditions, Eriksson (*39*) treated maize plants in a gamma field for 50 days until anthesis. The rates of exposure ranged from 5 to 200 R/day. The control frequency of forward mutant *wx* pollen grains was 2.5×10^{-4} . At the lowest exposure rate, 5 R/day, the frequency of mutant pollen grains increased to 7.9×10^{-4}. A linear dose-response curve was observed for exposure rates of 5 to 87 R/day. The frequency of mutant pollen grains increased much more rapidly at the higher exposure rates, 130 to 0 R/day.

The general interpretation of linear dose-response curves at low mutagen exposures is that such a

response indicates one-hit events or point mutations or minute deletions. An increased slope due to higher doses is interpreted as due to two-hit events or chromosome aberrations.

The above studies on forward mutation at the *wx* locus have demonstrated that acute and chronic gamma radiation induces a linear increase in mutation frequency with increased dose. The effect of x-radiation on reversion frequencies of three *wx* heteroalleles, *wx-H21*, *wx-C*, and *wx-90* , and on the frequency of intragenic recombination among three different heteroallelic combinations has been reported by Briggs and Smith (*45*). They found an increase in the reversion frequency of the *wx-90* heteroallele and a significant decrease in intragenic recombination between the *wx-C* and *wx-90* heteroalleles. Bianchi (*46*) analyzed the effect of increased x-radiation (0 to 1680 R) on plants heteroallelic for the following genotypes: *wx-H21/wx-90*, *wx-C/wx-H21*, and *wx-90/wx-C*. The induction of *Wx* pollen grains was scored. Only the *wx-H21/wx-90* heteroallelic combination showed a significant increase in the frequency of *Wx* pollen grains. However, a direct dose-dependent response for the induction of aborted pollen grains was observed in all genotypes. Of great interest was the fact that in homoallelic plants for *wx-C* or *wx-90*, an increase in revertant *Wx* pollen grains over the spontaneous frequency was demonstrated. For *wx-C* the exposure of 0, 800 R, and 1600 R of x-rays gave frequencies of revertant pollen grains of 1.2, 7.5, and 5.0×10^{-5}, respectively. For *wx-90* the same radiation doses induced frequencies of revertant pollen grains of 2.3, 5.6, and 11.5×10^{-5}, respectively. Thus *wx-C* and *wx-90* can revert to the dominant allele after exposure to a mutagen.

The analysis of maize pollen grains for forward or reverse mutation at the *wx* locus is based on the fact that a pollen grain carrying a *Wx* allele synthesizes the carbohydrate amylose while a pollen grain carrying the *wx* allele does not (*27*, *28*). A pollen grain carrying a *Wx* allele will turn black with the gelatin-iodine stain while a pollen grain carrying a *wx* allele will stain a tan color. Slides of pollen grains that were used in the following studies were prepared by a procedure developed by Nelson (*34*) and modified by Plewa (*47*).

In the reverse mutation tests plants homozygous for a specific *wx* allele were used. After treatment the tassels were harvested and the pollen grains analyzed. In a field of pollen grains carrying a *wx* allele exceptional pollen grains that carry the dominant *Wx* allele were scored. As illustrated in Figure 2 a black-staining pollen grain (*Wx*) is indicated among numerous tan-staining pollen grains (*wx*). It is assumed that a pollen grain from a homozygous *wx* sporophyte that has acquired the ability to synthesize amylose resulted from a reverse mutation at the *wx* locus. However, suppressor mutations may also produce phenotypically "revertant" pollen grains. The number of revertant pollen grains and an estimate of the total number of viable pollen grains were determined. In the forward mutation test plants homozygous for the dominant allele, *Wx*, were used. After treatment, harvesting and slide preparation the slides were analyzed for forward *wx* mutants. The specific genetic alteration that caused the inability of a pollen grain to synthesize amylose may have been a point mutation within the *wx* cistron, a deletion in or of the *wx* cistron, a chromosome aberration that resulted in a deficiency that included the *wx* locus or a chromosome aberration that induced a position effect that repressed the expression of the *Wx* allele. Finally, the possibility exists that a mutation resulted in a regulatory gene involved in the control of amylose synthesis. In this assay, tan-staining *wx* mutants were scored among a field of black-staining *Wx* pollen grains (Fig. 3). Although the forward mutation assay is not as specific as a reversion test, it provides a measurement of genetic damage in the sense that it involves a single locus.

In both the reverse and forward test procedures, the frequency of clear, collapsed, aborted pollen grains was also determined. The frequency of mutant pollen grains was determined by first scanning the entire microscope slide with a stereomicroscope at a magnification of 40 × and counting every exceptional pollen grain. The number of viable and aborted pollen grains on the slide was estimated by counting pollen grains within 20 randomly distri-

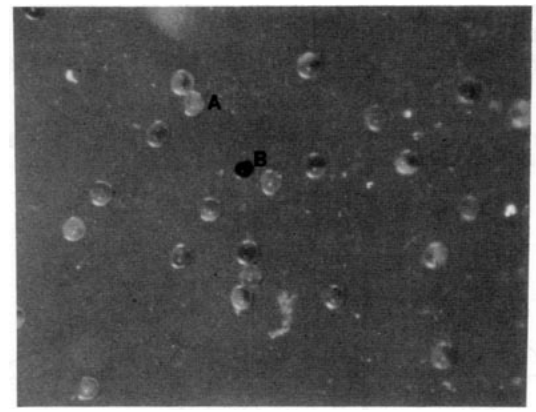

FIGURE 2. Reverse mutation at the *wx* locus in maize pollen grains: (*A*) a pollen grain carrying the recessive *wx-C* allele; (*B*) a revertant (reverse mutant) pollen grain; (*C*) an aborted pollen grain. The pollen grains are from a plant of inbred M14 homozygous for the recessive wx-C allele.

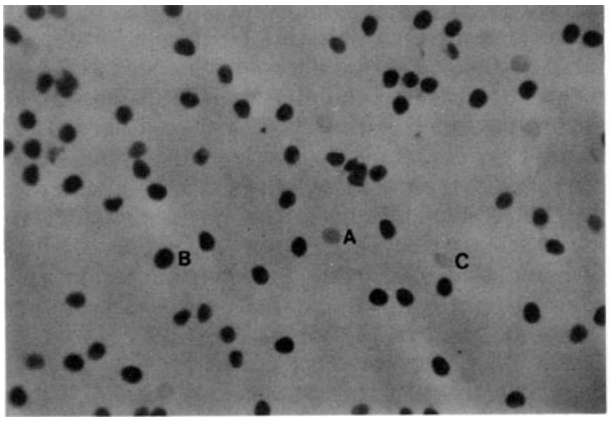

FIGURE 3. Forward mutation at the *wx* locus in maize pollen grains: (*A*) a forward mutant pollen grain; (*B*) a pollen grain carrying the dominant *Wx* allele; (*C*) an aborted pollen grain. The pollen grains are from a plant of inbred Early-Early Synthetic that was homozygous for the dominant *Wx* allele.

buted 1 mm^2 areas and multiplying by an appropriate factor. Thus, the data from each slide included the number of mutant pollen grains, the estimated number of viable pollen grains and the estimated number of aborted pollen grains.

Statistical Tests

Statistical tests were used to describe the distribution of the frequencies of mutant pollen grains and to determine the level of significance among the groups within an experimental design. Parametric statistics such as the mean, standard error of the mean, and the analysis of variance were used to detect significant differences between the frequencies of mutant pollen grains in control and experimental (i.e., treated) plants. The frequency of mutant pollen grains was determined for each plant by dividing the total number of mutant pollen grains by the estimated number of viable pollen grains derived from the slides of a single tassel. The population of pollen grains analyzed was kept approximately the same for each tassel. The mean frequency of mutant pollen grains was determined as the average of the frequencies of mutants of each tassel within a control or treatment group. The standard error of the mean for the tassels was determined. The standard error was defined as $SD/\sqrt{N}$, where SD is the standard deviation and N is the total number of viable pollen grains (*48*). An analysis of variance was conducted to determine if a significant difference existed among the mean frequency of mutant pollen grains in the control and the mean frequency of mutants in the various treatment groups. We regarded the mean frequency of mutant pollen grains in the control groups as representative of the spontaneous frequency of mutants.

Another test statistic (ϕ) that we used to determine if a treatment induced a significant increase in the frequency of mutant pollen grains was developed by Katz (*49*). He adapted the ϕ statistic for use in the analysis of mutation data from pollen grains (*50*). The test is defined as:

$$\phi = \frac{n(M - 0.5) - N(m + 0.5)}{\sqrt{nM(m + M)}}$$

where m is the observed number of mutant pollen grains in the control group, M is the observed number of mutant pollen grains in the experimental group, n is the estimated number of pollen grains analyzed in the control group and N is the estimated number of pollen grains analyzed in the experimental group. If a one-tailed test is performed and if the level of significance is set at 0.05 a mutagenic response is demonstrated if $\phi > 1.64$.

In Situ Tests

Evaluation of Pesticides

We have conducted a comprehensive evaluation of the mutagenic properties is 32 pesticides or combination of pesticides used in commercial corn production. The tests were conducted *in situ* under modern agricultural conditions and in the laboratory using both field grade and technical grade formulations. The laboratory studies included the evaluation of each pesticide or combination for the induction of mutation in *Salmonella typhimurium* and gene conversion in *Saccharomyces cerevisiae*. Each compound was assayed directly, with S9 *in vitro* mammalian microsome activation and with maize 1S *in vivo* plant activation protocols (*6, 51, 52*). In this paper we shall only discuss the *in situ* experimental design where reversion at the *wx-C* locus in maize pollen grains was measured to determine the presence of an environmental mutagen. The purpose of this discussion is to outline procedures that insure an adequate experimental design for *in situ* evaluations of environmental mutagens.

A separate herbicide test plot and an insecticide test plot were constructed. The test plots were divided into subplots with the dimensions of 10 m in length and 3 m in width. Control subplots were distributed within each test plot. The middle row of each subplot was planted with seven kernels of inbred W22 homozygous for the *wx-C* allele. The

outer rows of each subplot were planted with commercial hybrid corn to simulate field conditions. The appropriate pesticide or combination of pesticides was applied separately to each subplot prior to emergence of the seedlings. After the plants reached early anthesis, tassels from each *wx-C/wx-C* plant were harvested, labeled and stored in 70% ethanol. The tassels were agitated several times in clean ethanol to wash away contaminant *Wx* pollen from the commercial varieties of corn. To insure that only pollen from inbred W22 was analyzed, unopened florets were removed from the tassels and rinsed in clean ethanol. The anthers were dissected from these florets, the pollen grains were suspended in the gelatin-iodine stain and slides were prepared for analysis.

Data from the *in situ* pesticide tests are presented in Tables 1, 2, and 3. The control data are from subplots in the herbicide test plots, subplots in the insecticide test plot and a control plot on virgin uncultivated soil. The summed control values based on three years of studies indicate a frequency of revertant pollen grains of 4.64×10^{-5}. However, we compared the control values for each test plot with the values for each pesticide treatment within the same test plot. Several pesticides induced mutation where at least a doubling in the frequency of revertants over control values was observed. In Table 1 the data indicate that most treatments with *s*-triazine herbicides increased the frequency of revertant pollen grains. The herbicides that induced a mutagenic response were cyanazine, SD50093 (a formulation of atrazine + cyanazine) and procyazine + Dual. Slightly higher frequencies of revertants were observed in the treatments of cyanazine + Lasso and propachlor + cyanazine. The data from the insecticide test plot indicate that chlordane, Dyfonate and heptachlor induced higher frequencies of revertants. In Tables 2 and 3, data from additional *in situ* studies on the *s*-triazine herbicides are presented. The ϕ statistic was used to test for significant differences between the control frequency of mutant pollen grains and the revertant frequencies from plants treated with simazine, atrazine, cyanazine, and SD50093. All four *s*-triazine herbicides indicated a positive mutagenic response with

Table 1. Reverse mutation at the *wx* locus in maize pollen grains.

Treatment or pesticide	Application rate, kg/ha	Gametophytes analyzed	Frequency of revertants $\times 10^{-5}$
1976 Fields			
Herbicides controls	0	2,588,598	5.56 ± 0.98
Cyanazine	3.58	471,235	28.23 ± 3.36
Lasso	—	—	—
Modown	2.24	1,386,323	9.05 ± 0.87
SD50093	4.48	373,437	13.65 ± 0.23
Eradicane	0.56	537,822	2.23
Eradicane	3.36	391,310	7.67
Cycle	3.58	241,015	4.65 ± 1.76
Dual	6.00	959,329	4.32 ± 1.14
Propachlor	—	—	—
Cyanazine + Lasso	2.24 + 2.24	958,039	9.93 ± 3.89
Lasso + Modown	2.34 + 1.12	1,383,846	6.67 ± 0.84
Lasso + Modown	2.24 + 1.68	1,531,552	4.42 ± 0.32
Lasso + Banvel	2.34 + 0.56	1,438,457	4.02 ± 0.57
Propachlor + Cyanazine	3.36 + 2.24	246,927	9.31
Dual + Banvel	2.24 + 0.56	461,712	8.87 ± 1.12
Cycle + Dual	2.24 + 2.24	675,314	11.63 ± 1.91
Insecticide controls	0	1,991,435	4,16 ± 1.10
Chlordane	2.24	854,517	13.69 ± 1.10
Counter	2.24	999,857	7.40 ± 2.35
Dyfonate	2.24	727,776	10.12 ± 2.47
Heptachlor	1.12	730,461	10.87 ± 3.06
Thimet	2.24	1,495,862	3.75 ± 0.64
CGA-20G	2.24	1,248,182	6.74 ± 0.24
Furadan	2.24	1,477,373	6.15 ± 1.07
Lorsban	2.24	1,426,157	2.91 ± 1.73
Mocap	2.24	1,566,059	8.27 ± 2.44
SRA-15G	2.24	1,604,867	6.86 ± 1.52
Virgin soil controls	0	1,812,137	4.78 ± 0.10
Summed controls based on three years of field studies	0	8,649,485	4.64 ± 0.38

$p < 0.001$. Simazine has been listed as a neoplastic agent (*53*).

From these studies we concluded that the *wx* locus in maize pollen grains was a useful and sensitive assay for environmental mutagens under *in situ* conditions. The advantages of the assay were that the plants were able to chronically monitor a discrete environment for the presence of mutagens, the tassels could be stored indefinitely after the treatment and analyzed when convenient, the plants were easily incorporated into an agricultural setting, the assay was relatively rapid for a higher eukaryote and the large populations of pollen grains analyzed provided a good basis for the statistical interpretation of the data.

Evaluation of Municipal Sewage Sludge

We have begun a second *in situ* study and have improved our methods of environmental monitoring by virtue of our experience with the pesticide test plots. An attractive method of disposal of sludge resulting from the treatment of municipal sewage is the application of this organic matter to agricultural lands or its use in reclaiming land disturbed by surface mining. We are assaying sludge from the Calumet and Southwest plants of the Metropolitan Sanitary District of Chicago for the presence of mutagenic agents. This study has a variety of prokaryote and eukaryote genetic endpoints, however, in this paper we shall only present the experimental design for the *in situ* tests involving maize. A test plot was constructed on the NW900 plots at the University of Illinois Agronomy Research Center near Elwood, Illinois. A history of the use of these plots is presented in Table 4. It is important to know the past conditions of the area that is to be monitored. We were especially interested to know of the past use of pesticides on the test plots. The NW900 plots had four levels of sludge application in 1979 (Fig. 4). The control plot, Number 13, received no sludge application and adequate nitrogen was provided by the application of chemical fertilizer. The maximum treatment plot, Number 5, re-

Table 2. Reverse mutation at the *wx* locus of maize pollen grains.

Treatment or pesticide	Application rate, kg/ha	Gametophytes analyzed	Frequency of revertants $\times 10^{-5}$
1977 Fields			
Control	0	1,061,239	3.13 ± 0.49
Simazine	3.84	818,504	12.00 ± 2.31
Atrazine	3.84	950,194	8.92 ± 1.92
Sutan	7.20	908,052	6.42 ± 1.21
Sutan + Cyanazine	4.80 + 2.40	1,017,918	5.76 ± 1.19
Sutan + Atrazine	4.80 + 1.92	1,236,895	8.75 ± 1.16
Dual + Cyanazine	4.80 + 4.80	516,645	11.08 ± 3.11
Dual + Atrazine	3.00 + 2.40	791,308	12.27 ± 2.34
Eradicane + Atrazine	3.60 + 1.92	886,337	10.77 ± 1.59
Eradicane + Cyanazine	3.60 + 2.40	883,220	9.18 ± 1.46
1977 Fields			
Control	0	1,061,239	3.13 ± 0.49
Cyanazine	4.80	941,699	14.76 ± 2.22
SD50093	4.80	1,089,942	15.54 ± 3.09
Eradicane EPTC	7.20	808,937	5.31 ± 1.64
Lasso	6.00	1,354,070	4.49 ± 0.75
Propachlor + Cyanazine	4.80 + 2.24	1,160,786	7.06 ± 2.19
Dual + Banvel	3.00 + 0.60	752,178	7.40 ± 1.69

Table 3. Reverse mutation at the *wx* locus of maize pollen grains: mutagenicity of s-triazine herbicides in *Zea mays* inbred W22.

Pesticide	*Field grade application* rate, kg/ha	*Frequency of mutant* pollen grains ($\times 10^{-5}$)	*Statistical significance* ϕ^a	p
Control	0	33/1,061,239 (3.11)	–	–
Simazine	3.84	97/818,504 (11.85)	7.05	<0.001
Atrazine	3.84	81/950,194 (8.53)	5.00	<0.001
Cyanazine	4.80	141/941,699 (14.97)	8.91	<0.001
SD50093	4.80	150/1,089,942 (13.76)	8.39	<0.001

[a]This statistical test is adapted from Katz (*50*).

Table 4. History of the NW 900 plots at the University of Illinois Agronomy Research Center.

Year	Treatment
1968	Plots received 61.06 mt/ha sludge solids.
1969	Plots planted to kenaf, received 41.48 mt/ha sludge solids.
1970	0.5 gl Eptam 6E was applied and disced into the soil and alfalfa was seeded; 32 g dalapon (Dowpon) in 1.5 gal water was applied to the sludge plots; plots received 28.38 mt/ha sludge solids
1971	No sludge applied; plots in alfalfa and Dowpon sprayed on grassy areas for grass control
1972	Plots in alfalfa; plots received 12.16 mt/ha sludge solids
1973	Plots in alfalfa; plots received 20.77 mt/ha sludge solids
1974	No crop; plots received 46.21 mt/ha sludge solids
1975	No sludge applied; plots in alfalfa
1976	No sludge applied; plots in spinach
1977	No sludge applied; plots in spinach
1978	No sludge applied; plots in spinach
1979	Plots Numbers 5, 13, 14, and 15 used in the maize *wx* locus assay

ceived 17.8 cm of liquid sludge which was approximately 21.0 mt/ha of dry material. The one-half treatment plot, Number 15, received 8.9 cm of liquid sludge which was approximately 10.5 mt/ha of dry material. The one-fourth maximum treatment plot, Number 14, received 4.5 cm of liquid sludge which was approximately 5.0 mt/ha of dry material. The construction of the NW900 test plots and the application of the sludge was supervised by Dr. T. Hinesly of the Department of Agronomy at the University of Illinois.

Two lines of the maize inbred M14 were used, and the genotype of each line was *wx-C/wx-C* and *wx-90/wx-90*. Thirty kernels of each line were planted in each plot and the sporophytes grown to early anthesis at which time the tassels were harvested. The pollen was analyzed by Dr. S. Wood of the Institute for Environmental Studies at the University of Illinois. Although the data are currently being evaluated there appears to be a direct dose-dependent effect with an increased percentage of aborted pollen grains in the lines homozygous for the *wx-C* allele in the sludge treated plots. However, no increased frequency of revertant pollen grains over the control frequency was noted in any treatment group. A different response was observed in the lines that were homozygous for *wx-90*. There was no significant difference in pollen abortion among the control and treatment groups, however, tassels from the maximum treatment plot had a higher frequency of revertant pollen grains than the control. Additional tests are in progress.

The examples outlined in this section were presented to indicate the experimental designs that have been conducted to evaluate the practical use of maize as an environmental monitor. The requirements to make an adequate *in situ* assay are: (1) the knowledge of the history of the area to be assayed, (2) the construction of test plots with adequate controls, (3) the use of defined inbreds with specific alleles and a sufficient population of plants per treatment group, (4) personnel to tend the test plots and harvest the tassels, (5) the careful and competent analysis of the pollen grains, and (6) proper statistical evaluation of the data.

Inbred Early-Early Synthetic

During the last few years we evaluated a number of rapidly maturing maize varieties for use in studies on environmental mutagenesis. A useful variety, Early-Early Synthetic was developed by Dr. D. E. Alexander of the Department of Agronomy at the University of Illinois. We have inbred this variety for five generations and have tested the plants under acute and chronic exposure to mutagens under laboratory conditions. Early-Early Synthetic is a rapidly maturing inbred that develops from kernel to tassel emergence in approximately 4 weeks in a plant growth chamber. The plant reaches a height of approximately 50 cm and can be grown in 10 cm diameter plastic pots. In a 0.92 m^2 plant growth chamber we easily accommodated 40 plants to maturity. A mature Early-Early Synthetic plant is illustrated in Figure 5.

We evaluated Early-Early Synthetic for its response to chronic exposure of low concentrations of ethyl methanesulfonate (EMS) or maleic hydrazide (MH). The genetic endpoint was forward mutation at the *wx* locus in pollen grains. An experimental design included 20 plants with five plants per treatment group. A control of five plants was included with each experiment. Kernels within an experiment were siblings; this reduced the genetic component in the variance of the data. One kernel was planted per pot and after the seedling reached the three-leaf stage of development the pot was enveloped in a plastic bag. The opening of the bag

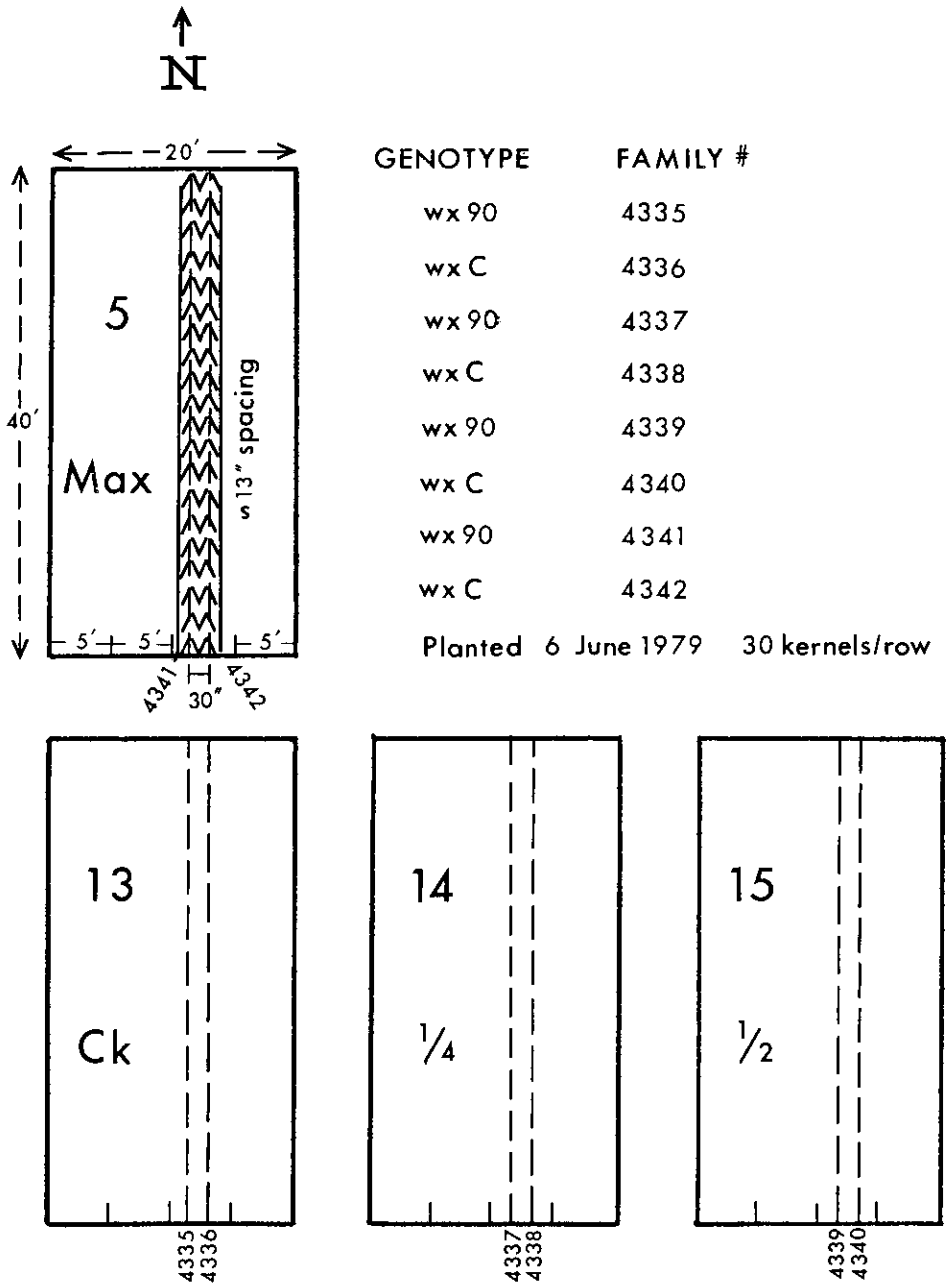

FIGURE 4. Outline of the NW900 test plots.

was secured around the stem of the seedling. The five pots in each treatment group were placed in a plastic tub in a growth chamber. The chamber lights were of mixed fluorescent and incandescent bulbs which provided 500 ft-candles of illumination at a distance of 15 cm below the lamps. The photoperiod was 14 hr, and the day and evening temperatures were regulated at 25 and 20°C, respectively. For each experiment the treatment groups were separated from the control group in a

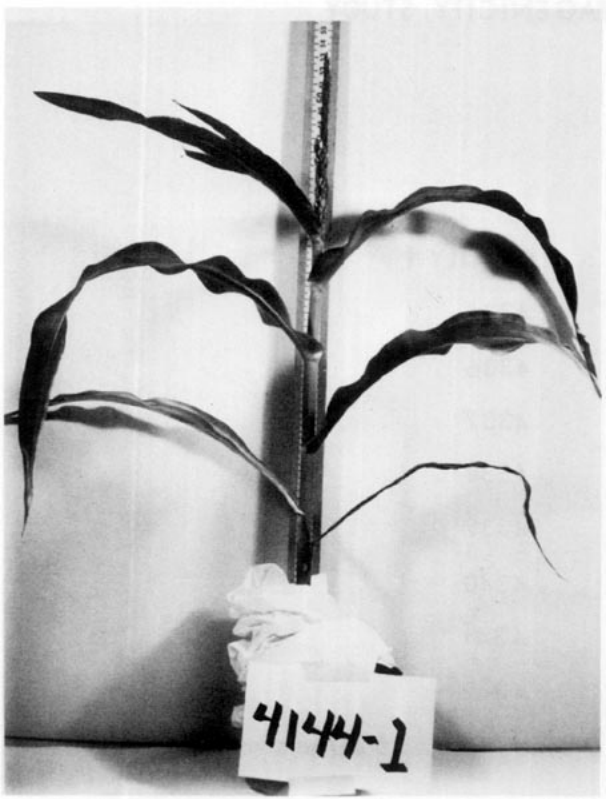

FIGURE 5. Inbred Early-Early Synthetic sporophyte.

sealed and locked plant growth chamber in an annex to our laboratory. The chamber that contained the treatment groups was kept at a time schedule that was advanced 12 hr from real time so the administration of mutagen solutions was conducted under darkened conditions. A plant growth chamber for the control groups was in our laboratory. All the plants were given identical amounts of water and fertilizer. The only variable was the concentration of mutagen in the treatment groups. The chemical mutagen, EMS or MH, was administered to the plants in an aqueous solution. The mutagen solution was prepared immediately prior to the treatment of the plants. A 50-ml portion of a known molar concentration was poured into the soil of each pot three times a week. The control plants received water only.

The data from the EMS experiments are presented in Table 5. This table is in a form that complies with the suggestions generated by the Plants Working Group of the USEPA Gene-Tox Program (*54*). Table 5 provides information on the molar concentration of EMS for each treatment group, the number of treatments or treatment days and the total exposure of EMS in moles per plant. The number of mutant pollen grains and the estimated number of viable pollen grains analyzed are presented as the frequency of mutant pollen grains. The percentage of pollen abortion was determined by estimating the number of aborted pollen grains and dividing by the sum of the viable plus aborted pollen grains. The percentage of pollen abortion is a measurement of gametophytic death and may have genetic as well as nongenetic causes.

In the four experiments the molar concentration of the EMS solutions ranged from 1 μM to 10 mM, and the total amount of EMS administered to individual plants ranged from 6.5×10^{-7} to 4.5×10^{-3} mole. Plants exposed to 6.0×10^{-5} mole during their sporophytic generation produced only aborted

Table 5. Chronic exposure of *Zea mays* inbred Early-Early Synthetic to ethyl methanesulfonate.

Expt. no.	Molar concn, μM	No. of days Treatment	EMS, moles/plant	Frequency of mutant pollen grains ($\times 10^{-5}$)	Pollen abortion, %	Statistical significance ϕ	Statistical significance p
4132-C	0	–	0	15/658,329 (2.28)	6.78	–	–
4132-3	100	12	6.0×10^{-5}	all aborted	100.00	–	–
4132-2	1000	12	6.0×10^{-4}	all aborted	100.00	–	–
4132-1	10,000	9	4.5×10^{-3}	all aborted	100.00	–	–
4133-C	0	–	0	4/502,024 (0.79)	9.04	–	
4133-3	1	13	6.5×10^{-7}	10/544,917 (1.84)	11.13	1.18	>0.05
4133-2	10	13	6.5×10^{-6}	35/535,457 (6.54)	6.13	4.60	<0.001
4133-1	50	12	3.0×10^{-5}	13/618,448 (2.10)	20.90	1.52	>0.05
4136-C	0	–	0	4/482,354 (0.83)	15.53	–	–
4136-3	10	14	7.0×10^{-6}	35/494,930 (7.07)	26.35	4.68	<0.001
4136-2	50	13	3.2×10^{-5}	16/291,002 (5.50)	36.45	3.68	<0.001
4136-1	100	14	7.0×10^{-5}	42/45,150 (93.02)	38.86	19.78	<0.001
				4 tassels all aborted	100.00		
4141-C	0	–	0	35/684,883 (5.11)	13.71	–	–
4141-3	2.5	9	1.1×10^{-6}	62/603,398 (10.28)	23.55	3.26	<0.001
4141-2	5	9	2.2×10^{-6}	88/644,892 (13.65)	8.29	5.03	<0.001
4141-1	15	9	6.8×10^{-6}	128/624,360 (20.50)	12.95	7.80	<0.001

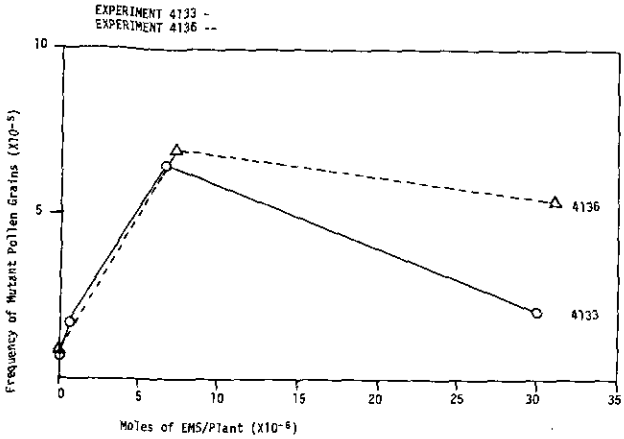

FIGURE 6. Dose-response curves for chronic exposure of Early-Early Synthetic maize to EMS. Experiments 4133 and 4136.

pollen grains (experiment 4132). Experiments 4133 and 4136 demonstrated that chronic exposure to EMS induced significant increases in the frequency of mutant pollen grains. This increased frequency was dose-dependent at total exposures of EMS below 1×10^{-5} moles per plant (Fig. 6). The total chronic exposure of EMS in experiment 4141 ranged from 1.1×10^{-6} moles to 6.8×10^{-6} mole (Table 5). The frequency of mutant pollen grains in every treatment group was significantly different ($p < 0.001$) from the control group and dose-dependent. Figure 7 illustrates the data from experiment 4141. The frequency of mutant pollen grains increased in a linear manner as a function of dose (coefficient of determination, $r^2 = 0.93$). However, there was no correlation between the exposure of EMS and the percentage of aborted pollen grains (coefficient of correlation, $r = 0.22$). Thus, EMS is a potent mutagen to Early-Early Synthetic when chronically administered to the soil.

In Table 6 we present data which demonstrate chronic exposure of Early-Early Synthetic to maleic hydrazide (MH) induces forward mutation at the *wx* locus in pollen grains. The experimental design was the same as for the EMS treatments. The total chronic exposure of MH in experiment 4143 ranged from 5.0×10^{-9} to 5.0×10^{-7} mole. The frequency of

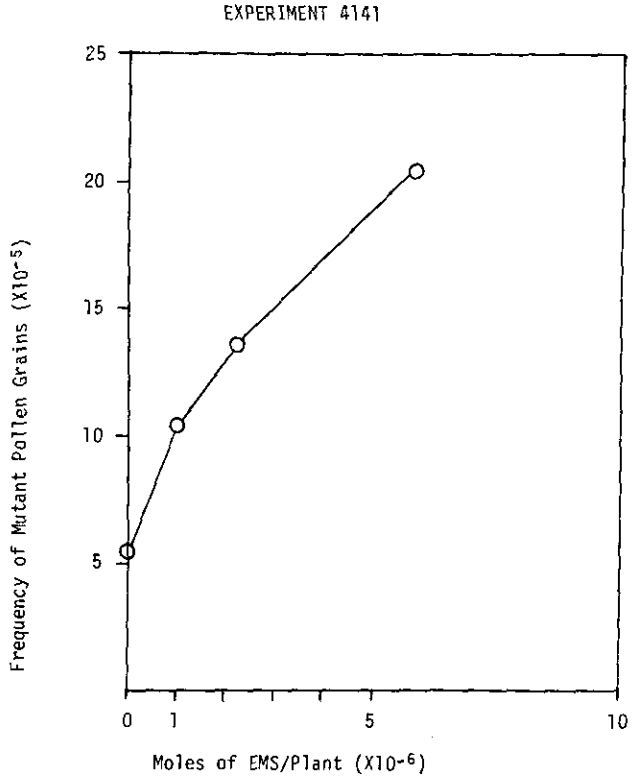

FIGURE 7. Dose-response curve for chronic exposure of Early-Early Synthetic maize to EMS. Experiment 4141.

mutant pollen grains ranged from 4.59×10^{-5} for the control group to 5.54×10^{-4} for the plants exposed to the highest concentration of MH. A statistically significant increase in the frequency of mutant pollen grains was observed in all treatment groups. This increase was dose-dependent and linear, $r^2 = 0.99$ (Fig. 8) The percentage of pollen abortion increased directly with the dose of MH ($r = 0.96$). These data clearly indicate that MH is an exceedingly potent mutagen in maize.

We chose MH as a test agent because this compound is a potent inducer of chromosome aberrations in many plant systems. However, MH is either ineffective or weakly active in bacterial mutation assays and in mammalian genetic and cytogenetic assays, reviewed by Haley (*55*); and

Table 6. Chronic exposure of *Zea mays* Inbred Early-Early Synthetic to maleic hydrazide.

Expt. no.	Molar concn, μM	No. of days Treatment	MH, moles/plant	Frequency of mutant pollen grains ($\times 10^{-5}$)	Pollen abortion, %	Statistical significance ϕ	Statistical significance P
4143-C	0	–	0	21/457,521 (4.59)	2.56	–	–
4143-3	0.0	10	5.0×10^{-9}	48/630,702 (7.62)	6.83	1.83	<0.04
4142-2	0.1	10	5.0×10^{-8}	95/593,399 (16.00)	5.98	5.44	<0.001
4143-1	1	10	5.0×10^{-7}	311/561,899 (55.35)	15.62	14.08	<0.001

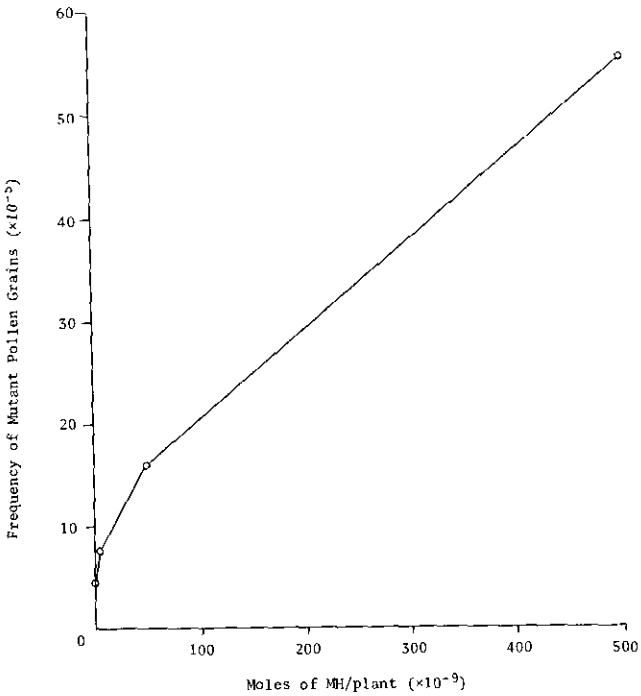

FIGURE 8. Dose-response curve for chronic exposure of Early-Early Synthetic maize to MH. Experiment 4143.

Swietlinska and Zuk (*56*). MH is a potent mutagen in maize (Table 6), and preliminary data suggests that this agent may be a plant promutagen that can be activated by a maize tissue homogenate into a form mutagenic in *S. typhimurium* strain TA98 (*57*).

Future Studies

For future studies we have incorporated the *yellow green-2 (yg-2)* and *dwarf (d)* alleles into Early-Early Synthetic. Plants that are heterozygous for *yg-2* can be used to detect mutation in seedling leaves (*58*). These plants could be used to measure germinal cell mutation in the pollen grains and somatic cell mutation in the leaves of the same sporophyte. Early-Early Synthetic plants homozygous for *d* would be very small, rapidly-maturing plants that would probably achieve a height of only 15-25 cm. These plants would be especially useful in laboratory studies or for *in situ* monitoring of enclosed areas. We shall investigate root-tip chromosomes for aberrations and sister chromatid exchanges as genetic endpoints for environmental monitoring. A procedure for sister chromatid exchange analysis in maize root-tip chromosomes has been developed by Chou and Weber (*59*). Finally, we shall continue the calibration of mutation induction in Early-Early Synthetic with a variety of chemical and physical mutagens.

In conclusion, the information presented demonstrates that *Z. mays* is a useful and sensitive genetic indicator organism. The plant has a number of genetic endpoints and can be used under laboratory or *in situ* conditions for studies in environmental mutagenesis.

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